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October 5, 1999

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Washington, DC 20231

Presented for filing is a new original patent application of:

Applicant: JAULANG HWANG, CHIA-TSE HSU AND
CHUN-JEN TING

Title: PEPTIDE REPEAT IMMUNOGENS

Enclosed are the following papers, including those required to receive a filing date under 37 CFR §1.53(b):

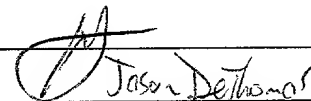
	<u>Pages</u>
Specification	13
Claims	3
Abstract	1
Declaration	2
Drawing(s)	3

Enclosures:

- Small entity statement. This application is entitled to small entity status.
- Assignment cover sheet and an assignment, 3 pages, and a separate \$40.00 fee.
- New disclosure information, including:
 - Information disclosure statement, 1 page.
 - PTO-1449, 1 page.
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Basic filing fee	380.00
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Respectfully submitted,



John T. Li
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Applicant or Patentee: Jaulang Hwang et al.
 Serial or Patent No.:
 Filed or Issued:
 For: PEPTIDE REPEAT IMMUNOGENS

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS
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I hereby declare that the nonprofit organization identified above qualifies as a nonprofit organization as defined in 37 CFR 1.9(e) for purposes of paying reduced fees under section 41(a) and (b) of Title 35, United States Code with regard to the invention entitled PEPTIDE REPEAT IMMUNOGENS by inventor(s) Jaulang Hwang, Chia-Tse Hsu and Chun-Jen Ting described in

- ☒ the specification filed herewith.
☐ application serial no. , filed .
☐ patent no. , issued .

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Title: Director, Office of Public Affairs and Technology Transfer, Academia Sinica

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Signature: Chi-Ming Liang

Date: 9/30/1999

APPLICATION
FOR
UNITED STATES LETTERS PATENT

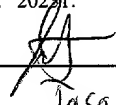
TITLE: **PEPTIDE REPEAT IMMUNOGENS**

APPLICANT: **JAULANG HWANG**
CHIA-TSE HSU
CHUN-JEN TING

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Jason DeThornos

PEPTIDE REPEAT IMMUNOGENS

Background of the Invention

5 The successful development of a protein-based vaccine often requires a delicate balance between enhancing immunogenicity of a particular antigen and the potential toxicity elicited by such enhancing. For example, an effective adjuvant used in animal studies (e.g., complete Freund's) may be too toxic to be used in vaccines prepared
10 for humans.

Summary of the Invention

 The invention is based on the discovery of a new means of generating an immune response to a peptide antigen
15 by concatemerizing the peptide and fusing the concatemer to a receptor binding domain of a *Pseudomonas* exotoxin. Such a fusion protein elicits antigen-specific antibodies in a variety of mammals, with little or no toxicity observed.

 Accordingly, the invention features a polypeptide
20 including (1) at least two copies (e.g., at least 12 or 16 copies) of a peptide sequence, and (2) a receptor binding domain of a *Pseudomonas* exotoxin A.

 The peptide sequence must be at least two amino acids in length (e.g., at least 3, 5, 7, 9, or 10 amino
25 acids in length). In a preferred embodiment, the peptide sequence can be less than 1000 amino acids in length (e.g., less than 500, 100, 50, or 20 amino acids in length). The peptide sequence can include any antigen in which an immune response against it is beneficial, such as gonadotropin
30 releasing hormone or GnRH (e.g., EHWSYGLRPG [SEQ ID NO:1]) or a fragment of a vaccinia virus coat protein (e.g., LIGICVAVTVAI [SEQ ID NO:2]).

A receptor binding domain is an amino acid sequence within a polypeptide which specifically binds to a LDL/alpha₂-microglobulin cell receptor. An example of a receptor binding domain is amino acids 1-252 (domain 1a) of the *P. aeruginosa* exotoxin A (PE):

5 mhliphwiplvaslgllaggssasaaeeafdlwnecakacvldlk
 dgvrrssrmsvdpaiadtngggvlhysmvleggndalklaidnals
 itsdgltirleggvepnkpvrtsytrqargswslnwlvpighekp
 snikvfihelnagnqlshmspiytiemgdellaklardatffvra
10 hesnemqptlaishagvsvmagtgprrekrwsewasgkvlclld
 pldgvyynylaqqrcnlddtwegkiyrv (SEQ ID NO:3).

Variants of the sequence immediately above, including substitutions, deletions, or additions are permissible, provided that the receptor binding domain specifically binds to a LDL/alpha₂-microglobulin cell receptor.

The position of the various elements within the polypeptide can be varied, as long the polypeptide is still able to elicit an immune response in a mammal. For example, all copies of the peptide sequence can be in a consecutive series, meaning that all copies occur as a single block of peptide repeats without intervening amino acids in between any two copies. In addition, the receptor binding domain can occur anywhere in the polypeptide (e.g., at the N-terminus, at the C-terminus, internal, or between two copies of the peptide sequence), as long as the intended function of the polypeptide as an antigen is not disrupted.

The invention also includes nucleic acids (e.g., expression vectors, including viral vectors) that encode a polypeptide of the invention. Such nucleic acids can be used in a naked DNA-based vaccines or to produce the polypeptides of the invention in large quantities.

In addition, the invention features a method of producing a polypeptide by (1) providing a nucleic acid of the invention, (2) introducing the nucleic acid into a cell

(e.g., a bacteria or eukaryotic cell, including cell lines and primary cells), and (3) expressing the polypeptide in the cell.

5 The polypeptides and nucleic acids of the invention can be used to provide new antigens and vaccines for eliciting an immune response against specific peptide sequences. These antigens can be formulated as safe and effective vaccines in mammals and humans, or at the very least, tested for efficacy in human and/or animal models, 10 including primate animal models.

Other features or advantages of the present invention will be apparent from the following drawings and detailed description, and also from the claims.

Brief Description of the Drawing

15 Fig. 1 is a representation of a GnRH peptide sequence (SEQ ID NO:1) useful in the polypeptides of the invention and primer sequences (SEQ ID NO:4 for Oligo A; SEQ ID NO:5 for Oligo B) which encode it or portions thereof.

20 Fig. 2 is a representation of how successive extension, denaturation, and annealing (i.e., PCR) of the primers in Fig. 1 produce a nucleic acid encoding concatamers of the peptide sequence.

Fig. 3 is a representation of a final PCR amplification of the concatemeric nucleic acid shown in 25 Fig. 2 to introduce at the ends of the concatemer a stop codon and suitable restriction sites for cloning. Adaptor primer A is designated SEQ ID NO:6. Adaptor primer B is designated SEQ ID NO:7. The 5' overhang sequence containing the EcoRI site is designated SEQ ID NO:8. The 5' overhang 30 sequence containing the SacII site is designated SEQ ID NO:9.

Detailed Description

The invention relates to new polypeptides having multiple copies of a peptide antigen fused to the receptor binding domain of a *Pseudomonas* exotoxin. Multiple copies of a peptide antigen is believed to increase immunologic presentation of epitopes present in each monomeric peptide sequence, though the mechanism is unclear. In addition, the exotoxin fragment is believed to increase binding between the polypeptide and antigen presenting cells, thereby facilitating uptake and presentation of peptide sequences.

I. Generation of Antibodies

The polypeptides of the invention can be used to generate antibodies that specifically bind a monomeric peptide sequence. Such antibodies can be used in diagnostic and/or therapeutic procedures that require the enhancement, inhibition, or detection of any molecule which contains the epitope presented by the peptide sequence.

In particular, various host animals can be immunized by injection of a composition containing a polypeptide of the invention. Host animals can include rabbits, mice, guinea pigs, and rats. Various adjuvants can be used to increase the immunological response, depending on the host species, including but not limited to Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanin, dinitrophenol, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and *Corynebacterium parvum*.

Antibodies include polyclonal antibodies, monoclonal antibodies, humanized or chimeric antibodies, single chain antibodies, Fab fragments, F(ab')₂ fragments, and molecules produced using a Fab expression library.

Monoclonal antibodies, which are homogeneous populations of antibodies to a particular antigen, can be prepared using the polypeptides described above and standard hybridoma technology (see, e.g., Kohler et al., Nature 5 256:495, 1975; Kohler et al., Eur. J. Immunol. 6:511, 1976; Kohler et al., Eur. J. Immunol. 6:292, 1976; Hammerling et al., In: Monoclonal Antibodies and T Cell Hybridomas, Elsevier, NY, 1981; U.S. Patent No. 4,376,110; Kosbor et al., Immunology Today 4:72, 1983; Cole et al., Proc. Natl. 10 Acad. Sci. USA 80:2026, 1983; and Cole et al., Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96, 1983). Such antibodies can be of any immunoglobulin class including IgG, IgM, IgE, IgA, IgD and any subclass thereof. The hybridoma producing the Mab of this invention 15 may be cultivated in vitro or in vivo. The ability to produce high titers of mAbs in vivo makes this an excellent method of production.

The antibodies can be used, for example, to detect the presence of an antigen in a biological sample as part of 20 a diagnostic assay, and also to evaluate the effectiveness of medical treatments by other therapeutic approaches.

In addition, techniques developed for the production of "chimeric antibodies" (Morrison et al., Proc. Natl. Acad. Sci. USA 81:6851, 1984; Neuberger et al., Nature 312:604, 25 1984; Takeda et al., Nature 314:452, 1984) by splicing the genes from a mouse antibody molecule of appropriate antigen specificity together with genes from a human antibody molecule of appropriate biological activity can be used. A chimeric antibody is a molecule in which different portions 30 are derived from different animal species, such as those having a variable region derived from a murine Mab and a human immunoglobulin constant region.

Alternatively, techniques described for the production of single chain antibodies (U.S. Patent Nos. 4,946,778, 4,946,778, and 4,704,692) can be adapted to produce single chain antibodies against a particular peptide antigen. Single chain antibodies are formed by linking the heavy and light chain fragments of the Fv region via an amino acid bridge, resulting in a single chain polypeptide.

Antibody fragments that recognize and bind to specific epitopes can be generated by known techniques. For example, such fragments include, but are not limited to, F(ab')₂ fragments that can be produced by pepsin digestion of the antibody molecule, and Fab fragments that can be generated by reducing the disulfide bridges of F(ab')₂ fragments.

II. Production and Use of Vaccine Compositions

The invention includes vaccine compositions (e.g., parenteral injectable vaccines) containing at least one polypeptide of the invention and, optionally, a pharmaceutically acceptable carrier, such as the diluents phosphate buffered saline or a bicarbonate solution (e.g., 0.24 M NaHCO₃). The carriers used in the composition are selected on the basis of the mode and route of administration, and standard pharmaceutical practice. Suitable pharmaceutical carriers and diluents, as well as pharmaceutical necessities for their use, are described in Remington's Pharmaceutical Sciences. An adjuvant, e.g., a cholera toxin, *Escherichia coli* heat-labile enterotoxin (LT), liposome, or immune-stimulating complex (ISCOM), can also be included in the new vaccine composition, if necessary.

The amount of vaccine administered will depend, for example, on the particular peptide antigen in the polypeptide, whether an adjuvant is co-administered with the

antigen, the type of adjuvant co-administered, the mode and frequency of administration, and the desired effect (e.g., protection or treatment), as can be determined by one skilled in the art. In general, the new vaccine antigens
5 are administered in amounts ranging between 1 μ g and 100 mg polypeptide per adult human dose. If adjuvants are administered with the vaccines, amounts ranging between 1 ng and 1 mg per adult human dose can generally be used. Administration is repeated as necessary, as can be
10 determined by one skilled in the art. For example, a priming dose can be followed by three booster doses at weekly intervals. A booster shot can be given at 8 to 12 weeks after the first immunization, and a second booster can be given at 16 to 20 weeks, using the same formulation.
15 Sera or T-cells can be taken from the individual for testing the immune response elicited by the vaccine against the neurotoxin. Methods of assaying antibodies or cytotoxic T cells against a specific antigen are well known in the art. Additional boosters can be given as needed. By
20 varying the amount of polypeptide, the copy number of peptide antigen in the polypeptide, and frequency of administration, the immunization protocol can be optimized for eliciting a maximal immune response.

Before administering the above compositions in
25 humans, toxicity and efficacy testing in animals are desirable. In an example of efficacy testing, mice (e.g., Swiss-Webster mice) can be vaccinated via an oral or parenteral route with a composition containing a polypeptide of the invention. For vaccines against an infectious agent,
30 after the initial vaccination or after optional booster vaccinations, the mice (and corresponding control mice receiving mock vaccinations) are challenged with a LD₅₀ dose of the infectious agent. End points other than lethality

can also be used. Efficacy is determined if mice receiving the vaccine die at a rate lower than the mock-vaccinated mice. Preferably, the difference in death rates should be statistically significant. Rabbits can be used in the above
5 testing procedure instead of mice.

Alternatively, the new vaccine compositions can be administered as ISCOMs. Protective immunity has been generated in a variety of experimental models of infection, including toxoplasmosis and Epstein-Barr virus-induced
10 tumors, using ISCOMS as the delivery vehicle for antigens (Mowat et al., Immunology Today 12:383-385, 1991). Doses of antigen as low as 1 μ g encapsulated in ISCOMs have been found to produce class I mediated cytotoxic T cell responses (Takahashi et al., Nature 344:873-875, 1990).

15 Without further elaboration, it is believed that one skilled in the art can, based on the above disclosure and the description below, utilize the present invention to its fullest extent. The following examples are to be construed as merely illustrative of how one skilled in the art can
20 practice the invention and are not limitative of the remainder of the disclosure in any way. Any publications cited in this disclosure are hereby incorporated by reference.

Example 1: A Multimeric Vaccine Against Gonadotropin
25 Releasing Hormone

Gonadotropin releasing hormone (GnRH) is a decapeptide produced by the arcuate nuclei of the hypothalamus and regulates expression of luteinizing hormone and follicle-stimulating hormone, which in turn regulates
30 gonad development in humans. In addition, increased expression of GnRH and its receptor has been correlated with a variety of tumors, including cancer of the breast, ovary,

endometrium, and prostate. See, e.g., Imai et al., Cancer 74:2555-2561, 1994; Eidne et al., J. Clin. Endocrinol. • Metab. 64:425-432, 1987; and Irmer et al., Cancer Res. 55:817-822, 1995. Therefore, antibodies against GnRH may
5 provide a means to modulate reproductive hormone activity and/or cancer development and progression.

To produce an antigen containing concatamers of GnRH fused to a receptor binding domain, the following approach, as outlined in Figs. 1-3, was used. This procedure was
10 designated template repeat PCR (TRPCR).

Two oligonucleotides were designed for TRPCR (Fig. 1). Oligo A encoded target antigen GnRH. Oligo B was complementary to oligo A. Oligo A is dissected into 5' half A1 and 3' half A2, both halves being of equal lengths.
15 Oligo B is dissected into 5' half B1 and 3' half B2, again both halves being of equal lengths. A1 was complementary to B1, while A2 was complementary to B2.

The thermal cycler was programmed for denaturation at 94°C for 30 seconds, annealing at 37°C for 30 seconds,
20 and extension at 72°C for 30 seconds. Using oligos A and B, PCR was performed for 30 cycles, followed by a final extension at 72°C for 10 minutes. This PCR should produced DNA species containing repeated sequences encoding GnRH, as illustrated in Fig. 2.

Two adapter primers (Adaptor A and Adaptor B) were designed to add an appropriate stop codon at the end of the repeated open reading frame (ORF) and two suitable restriction sites flanking the ORF (Fig. 3). For this step (which was designated adaptor PCR), the template for the PCR
25 was a 100-fold dilution of the TRPCR product produced as described above. The thermal cycler was programmed as described above, except that the denaturation was set for 1 minute instead of 30 seconds. The resulting PCR product
30

contained a SacII site at the 5' end, an EcoRI site at the 3' end, and a stop codon at the end of the ORF. •

The products of TRPCR and adaptor-PCR were then examined on a polyacrylamide gel. The majority of the TRPCR products were 500 bp to 700 bp in length. After adaptor PCR, products were distributed in a ladder, the lowest band containing the dimer of the GnRH DNA repeat and the slower migrating bands containing higher order multimers. The number of repeats present ranged from 3 to at least 12. One clone containing 12 repeats of GnRH coding sequence was chosen for further study.

The DNA fragment encoding 12 repeats of GnRH was subcloned into plasmid pPEDI, which was produced by subcloning the sequence encoding domain Ia of PE in pJH14 (Hwang et al., J. Biol. Chem. 264:2379-2384, 1989) into pET (Novagen). This plasmid expresses a polypeptide containing domain Ia of PE, which includes the toxin receptor binding domain of the toxin, and contains a His₆ tag at its N-terminus. The GnRH repeats were inserted at the 3' end of PE structure gene to produce pPEDIG12. The protein produced by this expression plasmid was designated PEIa-GnRH12.

pPEDIG12 was transformed into BL21(DE3)lysS. The transformants were cultured at 37°C in LB medium containing ampicillin (50 µg/ml), chloramphenicol (25 µg/ml) and tetracycline (10 µg/ml). When the A₆₀₀ of the culture reached 0.2, IPTG was added to the culture to medium to achieve a final concentration of 0.1 mM. Cells were cultured for another 90 minutes and then harvested. Since PEIa-GnRH12 is overexpressed in the form of inclusion bodies, the cells were extracted with 6 M urea.

The extracts were then purified using the Novagen pET His-Tag System. The fractions containing PEIa-GnRH12 were collected and dialysed against 50 mM ammonium acetate

(pH 4.0). PEIa-GnRH12 was stable for at least 6 months when stored at 4°C. After nickel-agarose affinity column chromatography, PEIa-GnRH12 was isolated to about 95% purity, as determined by SDS-PAGE.

5 To test this new immunogen, six week old New Zealand female rabbits were immunized with PEIa-GnRH12. A 0.5 ml bolus containing 100 µg of PEIa-GnRH12 and 125 µg aluminum phosphate (pH 7.0) was injected into the rabbit at the first time point (six weeks after birth). One week after the
10 first immunization, an identical 0.5 ml bolus was injected into the rabbits for a second immunization, followed by an identical injection two weeks after the first immunization. After the three immunizations, sera were collected for ELISA and immunoblotting analysis. To test whether antibodies
15 against the multimeric antigen was directed to monomeric epitopes or to epitopes spanning the junction between monomers, several GST fusion proteins containing various multimers of GnRH were produced as targets for ELISA.

 The antibodies produced in the immunized rabbits
20 failed to recognize with GST alone, while the sera at 5,000-fold dilution recognized all GST-GnRH fusion proteins to the same extent, irrespectively of how many GnRH repeats the GST fusion proteins contained. These results suggested that most of the antibodies produced in response to the immunogen
25 recognized monomeric GnRH epitopes rather than any hybrid epitopes created by concatemerization.

 GST-GnRH1 and GST-GnRH5 were specifically used as ELISA targets. Sera from thrice immunized rabbits, diluted 10,000-fold, recognized GST-GnRH1 and GST-GnRH5 to the same
30 extent, thereby confirming that the immune response against the immunogen was directed to GnRH monomer-specific epitopes.

To confirm that the antibodies elicited by the new immunogen was physiologically active, immunized rabbits were monitored for ovary development. As a control, rabbits were also immunized with PEIa-TopN8, a polypeptide containing domain Ia of PE fused to 8 repeats of a 10-amino acid topoisomerase N-terminal peptide. The PEIa-TopN8-immunized rabbits showed normal ovary development, while the ovaries of PEIa-GnRH12-immunized rabbits were significantly decreased in size. Thus, the GnRH immunogen produced can be used as an immunogen to induce autoantibodies useful for treating GnRH-associated diseases.

To further confirm the utility of the new GnRH immunogen, mice and a pig were also immunized with PEIa-GnRH12. The mice immunization regimen was identical to the rabbit immunization described above, except that each mouse received a 100 μ l bolus containing 10 μ g PEIa-GnRH12 and 25 μ g aluminum phosphate (pH 7.0) for each injection. In addition, a 24 day-old pig was injection once with a 1 ml bolus containing 10 mg PEIa-GnRH12 and 250 μ g aluminum phosphate (pH 7.0). GnRH-specific antibodies were readily elicited in the mice and pig, as well as in rabbits, indicating that the antigens can elicit an immune response in a variety of animals. Further, a single immunization was sufficient to elicit an immune response.

25 Example 2: A Multimeric Vaccine Against Vaccinia Virus

Instead of a GnRH peptide sequence, a DNA sequence containing a repeat of the vaccinia virus coat protein peptide LIGICVAVTVAI (SEQ ID NO:2) was constructed using PCR as described in Example 1 above. A PE fusion protein containing 16 repeats of the virus coat protein peptide was produced and purified according to the procedures in Example 1.

Six-week old rabbits were injected with a 1 ml bolus containing 100 μ g viral peptide repeat antigen and 100 μ g complete Freund's adjuvant. Four weeks later, the rabbits received a second 1 ml injection containing 100 μ g antigen and 100 μ g incomplete Freund's adjuvant. At 8 weeks after the first immunization, the rabbits were injected with a 1 ml bolus containing 100 μ g antigen (no adjuvant). Rabbits thrice immunized with the virus coat protein immunogen produced vaccinia virus-specific antibodies.

10 Thus, the general procedure of linking a peptide repeat to a PE receptor binding domain was shown to be successful for a second peptide.

Other Embodiments

15 It is to be understood that while the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of this
20 invention.

What is claimed is:

1 1. A polypeptide comprising (1) a receptor binding
2 domain of a *Pseudomonas* exotoxin A, and (2) at least two
3 copies of a peptide sequence.

1 2. The polypeptide of claim 1, wherein the peptide
2 sequence comprises a gonadotropin releasing hormone.

1 3. The polypeptide of claim 2, wherein the peptide
2 sequence comprises SEQ ID NO:1.

1 4. The polypeptide of claim 3, wherein all copies
2 of the peptide sequence are in a consecutive series.

1 5. The polypeptide of claim 2, wherein all copies
2 of the peptide sequence are in a consecutive series.

1 6. The polypeptide of claim 1, wherein the peptide
2 sequence comprises a fragment of a vaccinia virus coat
3 protein.

1 7. The polypeptide of claim 6, wherein the peptide
2 sequence comprises SEQ ID NO:2.

1 8. The polypeptide of claim 7, wherein all copies
2 of the peptide sequence are in a consecutive series.

1 9. The polypeptide of claim 6, wherein all copies
2 of the peptide sequence are in a consecutive series.

1 10. The polypeptide of claim 1, wherein the
2 polypeptide comprises 10 to 20 copies of the peptide
3 sequence.

1 11. The polypeptide of claim 10, wherein the
2 polypeptide comprises 10 to 20 copies of the peptide
3 sequence.

1 12. The polypeptide of claim 11, wherein all copies
2 of the peptide sequence are in a consecutive series.

1 13. The polypeptide of claim 1, wherein all copies
2 of the peptide sequence are in a consecutive series.

1 14. A nucleic acid encoding the polypeptide of
2 claim 1.

1 15. A nucleic acid encoding the polypeptide of
2 claim 3.

1 16. A nucleic acid encoding the polypeptide of
2 claim 6.

1 17. A nucleic acid encoding the polypeptide of
2 claim 10.

1 18. A nucleic acid encoding the polypeptide of
2 claim 13.

1 19. A method of producing a polypeptide, the method
2 comprising
3 providing the nucleic acid of claim 14;
4 introducing the nucleic acid into a cell; and
5 expressing the polypeptide in the cell.

1 20. A method of producing a polypeptide, the method
2 comprising

3 providing the nucleic acid of claim 15;
4 introducing the nucleic acid into a cell; and
5 expressing the polypeptide in the cell.

1 21. A method of producing a polypeptide, the method
2 comprising

3 providing the nucleic acid of claim 16;
4 introducing the nucleic acid into a cell; and
5 expressing the polypeptide in the cell.

1 22. A method of producing a polypeptide, the method
2 comprising

3 providing the nucleic acid of claim 17;
4 introducing the nucleic acid into a cell; and
5 expressing the polypeptide in the cell.

1 23. A method of producing a polypeptide, the method
2 comprising

3 providing the nucleic acid of claim 18;
4 introducing the nucleic acid into a cell; and
5 expressing the polypeptide in the cell.

PEPTIDE REPEAT IMMUNOGENS

Abstract of the Disclosure

The invention relates to a polypeptide including (1)
a receptor binding domain of a *Pseudomonas* exotoxin A, and
5 (2) at least two copies of a peptide sequence.

390417.B11

GnRH
Peptide sequence **E H W S Y G L R P G**

DNA sequence

	A 1	A 2
	GAACATTGGTCATAT	GGACTACGGCCGGGA
	CTTGTAACCAGTATA	CCTGATGCCGGCCCT
	B 1	B 2

Primer

	A 1	A 2
Oligo A	<u>GAACATTGGTCATAT</u>	<u>GGACTACGGCCGGGA</u>
Oligo B		<u>CCTGATGCCGGCCCT</u> <u>CTTGTAACCAGTATA</u>
	B 2	B 1

Fig. 1

Variable	Mean	SD	Min	Max	Skewness	Kurtosis	Normality
Age	35.2	12.5	18	65	0.15	3.2	0.98
Gender	1.2	0.4	1	2	0.05	3.0	0.99
Education	12.5	2.1	9	16	0.25	3.5	0.97
Income	15000	8000	5000	35000	0.35	3.8	0.96
Marital Status	1.8	0.4	1	2	0.05	3.0	0.99
Occupation	2.5	1.2	1	5	0.15	3.2	0.98
Health Status	1.5	0.5	1	2	0.05	3.0	0.99
Stress Level	3.2	1.5	1	5	0.25	3.5	0.97
Life Satisfaction	4.5	1.2	3	6	0.15	3.2	0.98
Resilience	2.8	1.0	1	4	0.25	3.5	0.97
Optimism	3.5	1.2	2	5	0.15	3.2	0.98
Emotional Stability	1.8	0.5	1	2	0.05	3.0	0.99
Self-Esteem	3.0	1.0	2	4	0.25	3.5	0.97
Life Purpose	2.5	1.2	1	4	0.15	3.2	0.98
Gratitude	3.8	1.0	2	5	0.15	3.2	0.98
Forgiveness	2.2	1.0	1	4	0.25	3.5	0.97
Resilience	2.8	1.0	1	4	0.25	3.5	0.97
Optimism	3.5	1.2	2	5	0.15	3.2	0.98
Emotional Stability	1.8	0.5	1	2	0.05	3.0	0.99
Self-Esteem	3.0	1.0	2	4	0.25	3.5	0.97
Life Purpose	2.5	1.2	1	4	0.15	3.2	0.98
Gratitude	3.8	1.0	2	5	0.15	3.2	0.98
Forgiveness	2.2	1.0	1	4	0.25	3.5	0.97

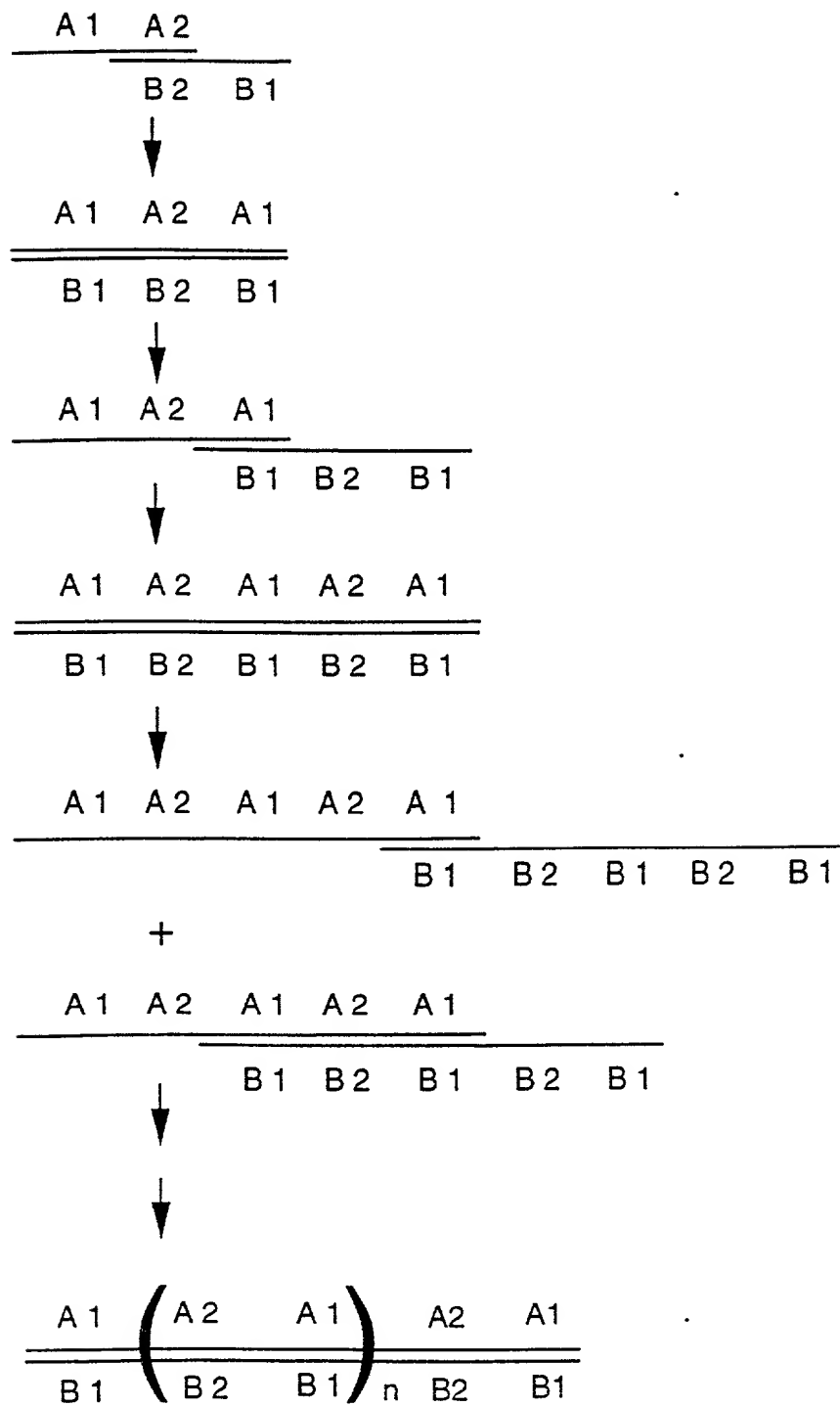
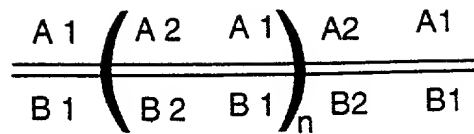


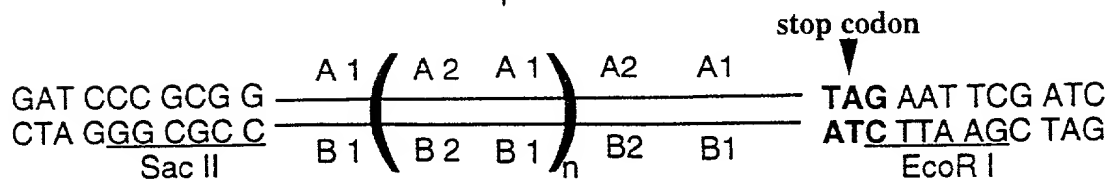
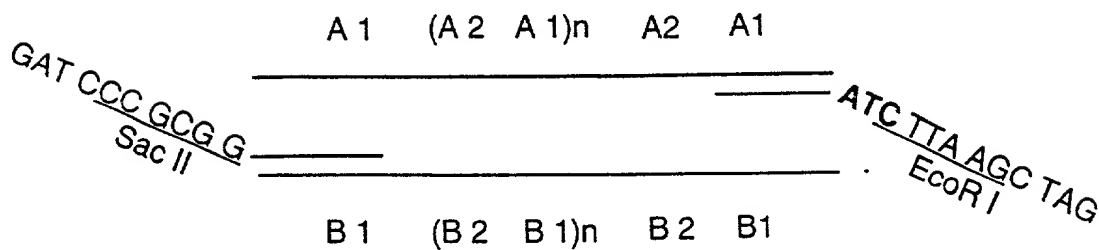
Fig. 3



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Adaptor A: GAT CCC GCG GCG AAC ATT GGT CAT ATG GA
Sac II

Adaptor B: GAT CGA ATT CTA ATA TGA CCA ATG TTC TCC
EcoR I



COMBINED DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled PEPTIDE REPEAT IMMUNOGENS, the specification of which ☒ is attached hereto.

- ☐ was filed on _____ as Application Serial No. _____ and was amended on _____.
- ☐ was described and claimed in PCT International Application No. _____ filed on _____ and as amended under PCT Article 19 on _____.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose all information I know to be material to patentability in accordance with Title 37, Code of Federal Regulations, §1.56.

I hereby appoint the following attorneys and/or agents to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith: Y. Rocky Tsao, Reg. 34,053; Eldora L. Ellison, Reg. 39,967; John F. Hayden, Reg. 37,640; John T. Li, Reg. 44,210; Frank R. Occhiuti, Reg. 35,306; Eric L. Prah, Reg. 32,590; and Gary A. Walpert, Reg. 26,098.

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patents issued thereon.

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